INHOMOGENEOUS TRANSLATIONAL DIFFUSION OF MONOCLONAL ANTIBODIES ON PHOSPHOLIPID LANGMUIR-BLODGETT FILMS

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ABSTRACT The translational mobility of fluorescent-labeled monoclonal antibodies specifically bound to supported phospholipid bilayers containing hapten-conjugated phospholipids has been measured as a function of the surface concentration of bound antibodies using fluorescence recovery after photobleaching. Fluorescence recovery curves are fit well by a model that assumes the presence of two populations of antibodies with different lateral diffusion coefficients. The larger diffusion coefficient equals 3.5×10^{-9} cm^2/s, the smaller diffusion coefficient ranges from 1.5×10^{-9} cm^2/s to 2.5×10^{-10} cm^2/s, and the fractional fluorescence recovery associated with the smaller coefficient increases from ~0 to ~0.7 with increasing concentration of bound antibody. These results suggest that complexes of haptenated phospholipids and antibodies in phospholipid Langmuir-Blodgett films form clusters or domains in a concentration-dependent fashion.

INTRODUCTION

The concentration dependence of macromolecular diffusion in solution is of current theoretical (O'Leary, 1987; Phillies, 1986) and experimental (Andries et al., 1983; Ullmann et al., 1985) interest, as is the concentration dependence of protein diffusion in two-dimensional biological membranes (Thompson et al., 1984; Hackenbrock et al., 1986; Beck, 1987). One method of obtaining precise measurements of the concentration dependence of protein lateral diffusion rates in or on two-dimensional fluid phospholipid bilayers is to employ Langmuir-Blodgett films. Supported phospholipid monolayers and bilayers have been used extensively as models of immunological cell membranes (McConnell et al., 1986; Thompson and Palmer, 1988). Langmuir-Blodgett films are also of interest in fields other than membrane biophysics, including protein crystallography, biosensors and bioelectronics, optics, and surface science (Uzgiris, 1986; Place et al., 1985; Zemel, 1983).

Antibodies specifically bind to Langmuir-Blodgett films containing hapten-conjugated phospholipids and the bound antibodies can be laterally mobile (McConnell et al., 1986; Subramaniam et al., 1985, 1986). Antibodies bound to phospholipid Langmuir-Blodgett films for several hours can also form planar crystals (Uzgiris and Kornberg, 1983; Uzgiris, 1986). The conditions under which bound antibodies are monomerically mobile, clustered, or crystallized are not yet well understood.

In the present work, the translational diffusion coefficients of bound antibodies have been measured as a function of the antibody surface concentration. At the lowest surface concentration, fluorescence recovery curves are described well by a model that assumes a single population of mobile, bound antibodies that diffuse with a rapid coefficient approximately equal to that of the lipids. At all other antibody surface concentrations this model is not adequate, but a model that assumes that diffusion is not homogeneous, i.e., that one population diffuses with a rapid coefficient and another population diffuses much more slowly, does describe the measured fluorescence recovery curves. Complete recovery is observed for all samples, but the fractional fluorescence recovery associated with the slowly mobile component increases dramatically with concentration. Extensive statistical analysis and control experiments support the choice of the two-component model rather than the one-component model for all but the lowest antibody surface concentration. These results suggest that complexes of haptenated phospholipids and antibodies in phospholipid Langmuir-Blodgett films can form clusters or domains by a concentration-dependent mechanism. Antibody clustering on cell surfaces may be important in immune function (McCloskey and Poo, 1984).

MATERIALS AND METHODS

Reagents

All water was purified to exceed standards for type I reagent grade water (NANOpure II, Barnstead, Sybron Corp., Boston, MA). Dimyristoylphosphatidylcholine (DMPC; Calbiochem-Behring Corp., La Jolla, CA) and 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxa-diazol-4-yl)amino]dodeca-

noyl]phosphatidylethanolamine (NBD-PE; Avanti Polar Lipids, Inc., Birmingham, AL) were used without further purification. The hapten-conjugated phospholipid DNP-TEMPO-DMPE shown in Fig. 1 was synthesized according to the method for "nitroxide lipid hapten II" of Balakrishnan et al. (1982*a*), except that dimyristoylphosphatidylethanolamine (Calbiochem-Behring Corp.) rather than dipalmitoylphosphatidylethanolamine was used as a starting reagent. AN02 hybridoma cells (a generous gift of Prof. H. M. McConnell of Stanford University) were maintained in culture, and the secreted monoclonal mouse IgG1 antibodies with specificity for DNP-TEMPO were purified from cell supernatants by protein A affinity chromatography as previously described (Balakrishnan et al., 1982*a*; Anglister et al., 1984). The purified AN02 antibody ran as appropriate bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. Specificity for DNP-glycine (Sigma Chemical Co., St. Louis, MO) was confirmed by tryptophan quenching as previously described (Anglister et al., 1984). Antibodies were labeled with fluorescein-5-isothiocyanate (Molecular Probes Inc., Eugene, OR) and the molar ratios of fluorescein to antibody (F:A) were calculated by the method of Mishell and Shiigi (1980).

Phospholipid Langmuir-Blodgett Films

Supported phospholipid bilayers consisting of DMPC, DMPC:DNP-TEMPO-DMPE (68:32, mol/mol), DMPC:NBD-PE (98:2, mol/mol), or DMPC:DNP-TEMPO-DMPE:NBD-PE (68:32:2, mol/mol/mol) were deposited on fused silica substrates as described (Tamm and McConnell, 1985), except that a Joyce-Loebl Langmuir trough (model 4, Vickers Instruments, Inc., Malden, MA) was employed for uniform compression speed (8.6 ± 0.1 Å^2/molecule min), coating speed (5.4 ± 0.2 mm/min), and coating pressure (35 dyn/cm). Supported bilayers, in the aqueous phase of the Langmuir trough, were mounted on clean glass slides in Teflon holders as shown in Fig. 2 *A*. The holders were designed to eliminate contact between the bilayers and materials other than Teflon. Bilayers were washed with 1 ml phosphate-buffered saline (PBS) (0.05 M sodium phosphate, 0.15 M sodium chloride, 0.01% sodium azide, pH 7.0) and treated with 100 μl of a PBS solution of fluorescent-labeled AN02 (F-AN02, F:A 0.6–1.4) at the specified concentration. The antibody solution for all samples except those prepared for gel electrophoresis and densitometry contained 0.65 mg/ml of unlabeled sheep IgG (Sigma Chemical Co.) to compete for nonspecific binding sites that occasionally may have been present on bilayers. In some experiments (as noted), the antibody solution contained DNP-glycine. After 20 min at room temperature, bilayers were washed with 3 ml PBS and transferred under PBS to the sample chamber for fluorescence microscopy shown in Fig. 2 *B*.

Fluorescence Microscopy

The fluorescence arising from antibodies bound to bilayers was measured on a fluorescence microscope, which consisted of an inverted optical microscope (Zeiss IM-35, Eastern Microscope Co., Raleigh, NC) and an argon ion laser (Innova 90-3, Coherent Inc., Palo Alto, CA) as previously

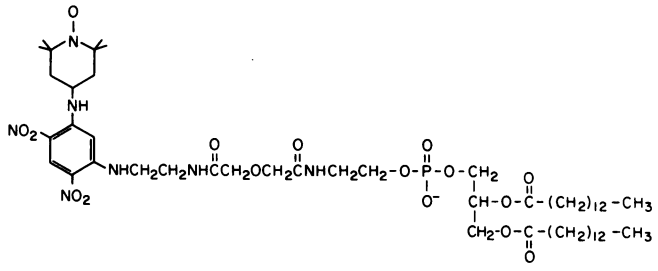

FIGURE 1 (DNP-nitroxide)-conjugated dimyristoylphosphatidylethanolamine.

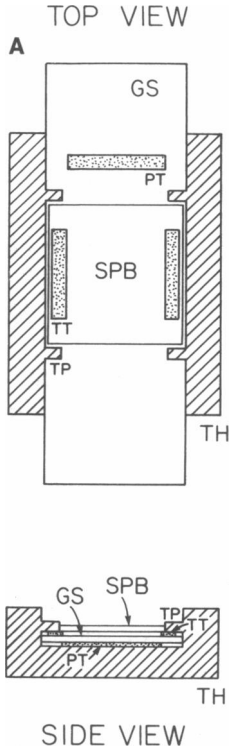

FIGURE 2 Apparatus for supported phospholipid bilayers. (*a*) Two strips of single-coated adhesive Teflon tape (*TT*, part No. S-15, Saunders Corp., Los Angeles, CA) were attached to one face of a clean glass slide (*GS*), and one strip of double-coated adhesive paper tape (*PT*) was attached to the other face. The slide was attached to a Teflon holder (*TH*) by the strip of double-coated tape and immersed in the aqueous phase of the Langmuir trough. Supported phospholipid bilayers (*SPB*) were gently rested on the strips of Teflon tape so that the bilayers faced the aqueous phase between the glass and fused silica slides and were held in place by four Teflon posts (*TP*). The assembly was then removed from the Langmuir trough. (*b*) SPB were transferred, in a large container of PBS, to a glass-bottomed petri dish of PBS. The dish was placed on a copper plate which was in thermal contact with a thermoelectric stage (model TS-4, Sensortek, Clifton, NJ) and brought to 30.5 ± 0.1°C. The assembly was mounted on the stage of the fluorescence microscope.

described (Palmer and Thompson, 1987). Translational diffusion coefficients were measured by fluorescence pattern photobleaching recovery (Smith and McConnell, 1978). The radii of the illuminated and observed circular areas in the sample plane, formed with a beam expander (5×, Oriel Corp., Stratford, CT) and an image plane aperture, respectively, were 160 and 75 μm. The ruling periodicity in the sample plane was 16.0 μm. Other photobleaching parameters were: excitation wavelength, 488.0 nm; objective, 40× 0.75 N.A.; observation and bleaching laser powers, 0.3–15 μW and 0.5 W; bleaching pulse duration, 25 ms–2 s; and depth of bleaching, 10–75%.

Gel Electrophoresis of Photobleached Antibodies

Bilayers consisting of DMPC:DNP-TEMPO-DMPE (68:32, mol/mol) that had been treated with 100 μg/ml F-AN02 (without sheep IgG) were translated through the laser beam under the same conditions used for the photobleaching pulse, at a rate such that the residency time in the illuminated area of an average bound antibody was 100 ms, using a computer-controlled microstepping microscope stage. Approximately 35% of the measured fluorescein fluorescence was bleached by this procedure. The bilayers were then transferred to the holders shown in Fig. 2 *A* and washed with 0.1% Triton X-100 detergent/H_2O, which removed ~ 60% of the bound F-AN02 as measured by fluorescence microscopy. The wash was analyzed on SDS-PAGE (7.5% acrylamide) under nonreducing conditions followed by staining with nickel (Kodavue, Eastman Kodak Co., Rochester, NY). The amount of collected F-AN02 that penetrated the acrylamide gel and ran as monomeric IgG was quantitated

by densitometry (Ultroscan XL, LKB Instruments, Inc., Gaithersburg, MD) using standards of known F-AN02 concentrations.

DATA ANALYSIS

For a sample containing one diffusive population of fluorophores, the normalized fluorescence pattern photobleaching recovery curve $\phi(t)$ is approximately given by (Smith and McConnell, 1978)

$$\phi(t) = \phi(0) + (\mu/2)[1 - \phi(0)]\{1 - (8/\pi^2) \cdot [\exp(-4\pi^2 Dt/a^2) - (1/9)\exp(-36\pi^2 Dt/a^2)]\} \quad (1)$$

for $t \geq 0$, where $\phi(t)$ is the ratio of the postbleach fluorescence $F(t > 0)$ to the constant prebleach fluorescence $F(t < 0)$, time zero is at the bleach pulse, μ and $1 - \mu$ are the fractions of the prebleach fluorescence that arise from mobile and immobile fluorophores, respectively, a is the ruling periodicity in the sample plane, D is the diffusion coefficient, and more rapidly decaying terms have been neglected. For $n \geq 1$ populations of mobile fluorophores and an additional population of immobile fluorophores,

$$\phi(t) = \phi(0) + \tfrac{1}{2}[1 - \phi(0)] \sum_{i=1}^{n} \mu_i\{1 - (8/\pi^2) \cdot [\exp(-4\pi^2 D_i t/a^2) - (1/9)\exp(-36\pi^2 D_i t/a^2)]\} \quad (2)$$

for $t \geq 0$, where μ_i is the fraction of the fluorescence recovery that arises from fluorophores that are mobile with coefficient D_i. Fluorescence recovery curves were fit to functions of the form of Eqs. 1 and 2 using an iterative Gauss-Newton algorithm, with μ, D, and $\phi(0)$ as free parameters for the "single mobile species" model ($n = 1$), μ_1, μ_2, D_1, D_2, and $\phi(0)$ as free parameters for the "two mobile species" model ($n = 2$), and μ_1, μ_2, μ_3, D_1, D_2, D_3, and $\phi(0)$ as free parameters for the "three mobile species" model ($n = 3$).

The significance of the improvement in the fit afforded by the n species model over the $n - 1$ species model was tested using an F statistic defined as

$$F_n = (\chi^2_{n-1} - \chi^2_n)(N - 2n - 1)/(2\chi^2_n), \quad (3)$$

where χ^2_n is the chi-squared goodness-of-fit statistic for the n mobile species model function and N is the number of data points. This F statistic has been used previously to discriminate between nonlinear model functions (Ratkowsky, 1983; Ameloot and Hendrickx, 1982). The probability distribution of the F statistic asymptotically approaches an exact F distribution if the errors in the experimental data are normally distributed and the number of data points approaches infinity. In the present work, the average number of photoelectrons per sample time was ~5,000 and the number of data points was ~600; therefore the distribution of the F statistic was expected to be close to an exact F distribution. This was confirmed by estimating the distributions of F_2 for the one and two mobile species models from sets of ~300 simulated recovery curves, which were generated by adding Poisson noise to the functions calculated from Eq. 2 with $n = 1$ or $n = 2$ using the average parameter values obtained from experimental data (Ratkowsky, 1983). Accordingly, the n species model was considered a significant improvement over the $n - 1$ species model for an experimental curve if the F statistic exceeded 3.00, which is the $P = 0.05$ critical value (the number greater than or equal to 95% of the F_n values which randomly occur for systems that actually contain $n - 1$ components) for an exact F distribution with degrees of freedom equal to 2 and ∞ (Devore, 1982). Similar considerations have been used in the analysis of multiexponential fluorescence lifetime decay curves (Ameloot and Hendrickx, 1982).

RESULTS AND DISCUSSION

F-AN02 bound specifically to DNP-TEMPO-DMPE in DMPC bilayers. As shown in Fig. 3, the fluorescence arising from bilayers incubated with F-AN02 required the presence of DNP-TEMPO-DMPE and increased with the solution concentration of F-AN02 with which bilayers were treated. The ratio of specifically bound F-AN02 to nonspecifically bound F-AN02 for the 100-μg/ml samples was ~250.

Soluble hapten (DNP-glycine) competed with the haptenated bilayer surface for F-AN02 binding sites. For bilayers treated with 100 μg/ml F-AN02 and increasing concentrations of DNP-glycine, the measured, background-corrected fluorescence decreased to zero within experimental uncertainty. The concentration of DNP-glycine at which the measured fluorescence intensity was decreased by a factor of 2 was 3.5×10^{-4} M, which

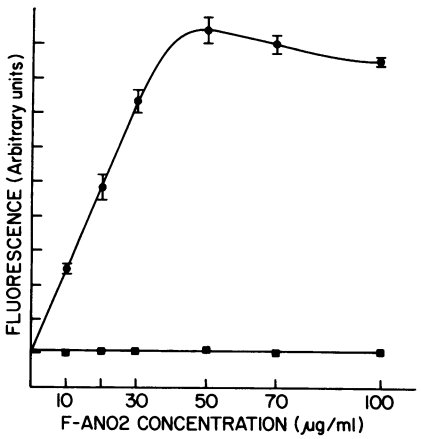

FIGURE 3 Antibody binding to supported phospholipid bilayers. Shown is the measured fluorescence from F-AN02 bound to bilayers composed of DMPC and 32 mol% (●) or 0 mol% (■) hapten-conjugated phospholipids as a function of the solution concentration of F-AN02 with which bilayers were treated. The mean values and standard errors of the mean for 15–30 measurements per antibody concentration are shown.

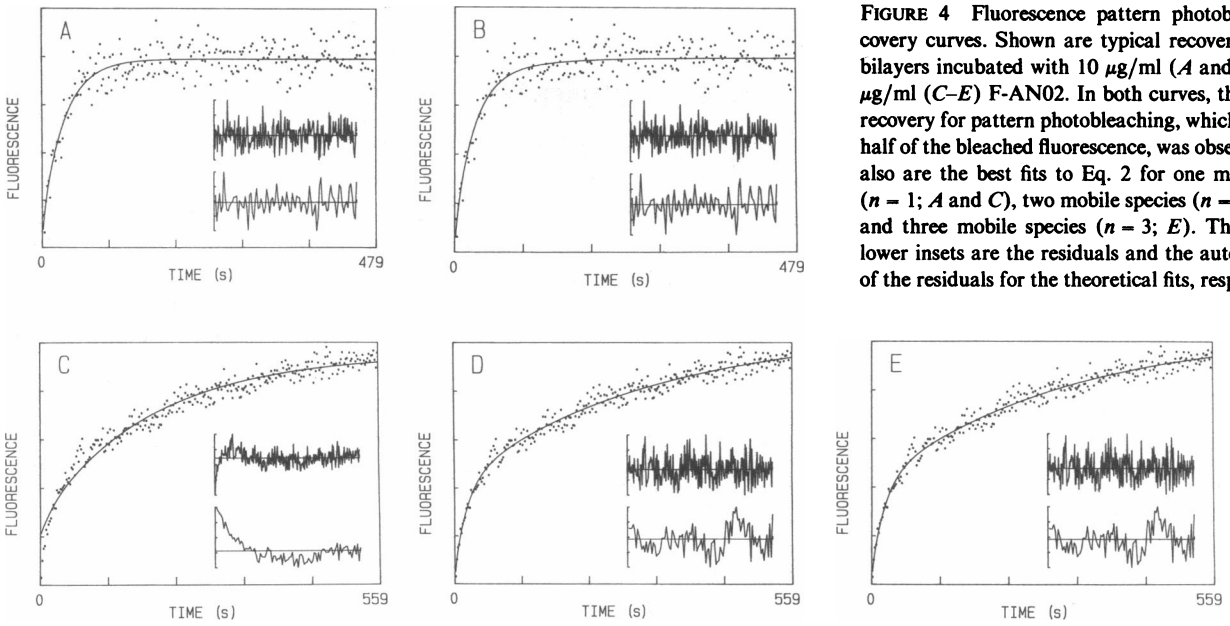

FIGURE 4 Fluorescence pattern photobleaching recovery curves. Shown are typical recovery curves for bilayers incubated with 10 μg/ml (*A* and *B*) and 100 μg/ml (*C–E*) F-AN02. In both curves, the maximum recovery for pattern photobleaching, which equals one-half of the bleached fluorescence, was observed. Shown also are the best fits to Eq. 2 for one mobile species ($n = 1$; *A* and *C*), two mobile species ($n = 2$; *B* and *D*) and three mobile species ($n = 3$; *E*). The upper and lower insets are the residuals and the autocorrelations of the residuals for the theoretical fits, respectively.

corresponds to an F-AN02 surface dissociation constant of 1 nM.[1]

Fluorescent-labeled, nonspecific sheep IgG did not bind to haptenated bilayers. The average background-corrected fluorescence intensity for haptenated bilayers treated with 100 μg/ml fluorescent-labeled sheep IgG (F:A, 0.23) relative to bilayers treated with 100 μg/ml F-AN02 (F:A, 0.30) was 0.09 ± 0.04.

Haptenated bilayers containing bound F-AN02 appeared uniformly fluorescent. The typical relative standard deviation from six measurements of fluorescence intensity on a single bilayer was 5–15%. Bilayer-to-bilayer variability was sometimes higher.

Fig. 3 demonstrates that bilayers containing different surface concentrations of bound antibodies can be reproducibly constructed. Removing the bound F-AN02 with detergent and calibrating the amount of removed F-AN02 with gel electrophoresis followed by densitometry yielded an F-AN02 surface concentration for the 100-μg/ml samples of ~5,000 molecules/μm². This number is consistent with the surface concentration of bound antibodies in the crystallized state (Uzgiris and Kornberg, 1983).

Specifically bound F-AN02 were mobile on supported bilayers with diffusion coefficients and fractional recoveries that depended on the concentration of F-AN02 with which bilayers were treated. Statistical analysis of the experimental data indicated that at the chosen critical value for the F statistic ($F_2 = 3.0$) two components of diffusion were present for all antibody concentrations except 10 μg/ml. Median values of F_2 were 2.0 for the 10-μg/ml F-AN02 samples, 8.6 for 20 μg/ml, and >50 for the remaining concentrations; therefore, with the exception of the 10-μg/ml F-AN02 samples, the values of F_2 were so large that the chosen critical value had little effect on the results. In contrast, the values of F_3, for comparison of two- and three-component models, were typically less than one, indicating that a third component was not statistically resolvable. Qualitative examination of typical experimental recovery curves, residuals, and residual autocorrelations shown in Fig. 4 supports the conclusion that the 10-μg/ml samples contain a single component and the higher concentration samples contain two components.

The average diffusion coefficients D_1 and D_2 and their fractional recoveries μ_1 and μ_2 from the best fits to functions of the form of Eq. 2 with $n = 2$ are shown in Fig. 5. If the two-component function did not significantly improve the fit (i.e., if $F_2 \leq 3.00$), D_1 and μ_1 were set equal to D and μ from the one-component fit (Eq. 1), μ_2 was set equal to zero, and D_2 was excluded from the mean. For all samples, maximum possible fluorescence recovery for pattern photobleaching (50%) was observed. The fraction of the recovery associated with the slow component varied from ~0 at 10 μg/ml to ~0.7 at 100 μg/ml. As shown in Fig. 3, the surface concentration of bound antibodies is approximately constant for incubation concentrations of antibodies >50 μg/ml, and explains the absence of change in the diffusion constant D_2 for concentrations >50 μg/ml in Fig. 5. The absolute fluorescence intensity ($\mu_1 F[t < 0]$)

[1]A simple model is assumed: $A + B \rightleftharpoons C$, where $K_1AB = C$, and $A + D \rightleftharpoons E$, where $K_2AD = E$, where *A–E* are the concentrations of antibody in solution, free surface sites, occupied surface sites, free soluble hapten, and antibody-hapten complexes in solution, respectively. Furthermore, $B + C = N$ and $h(E + A) + C = M$ are constants, where h is the thickness of the sample solution (~0.1 mm). These equations imply that $K_1 = h(1 + K_2D_{1/2})/(M - N/2)$, where $D_{1/2}$ is the concentration of soluble hapten at which $C = N/2$. The value of K_2 is $2 \times 10^6\ M^{-1}$ (Anglister et al., 1984). The value of N (5,000 molecules/μm²) is obtained from the calibration of bound antibodies at saturation (see text), and the value of M (40,000 molecules/μm²) is obtained from the antibody solution concentration.

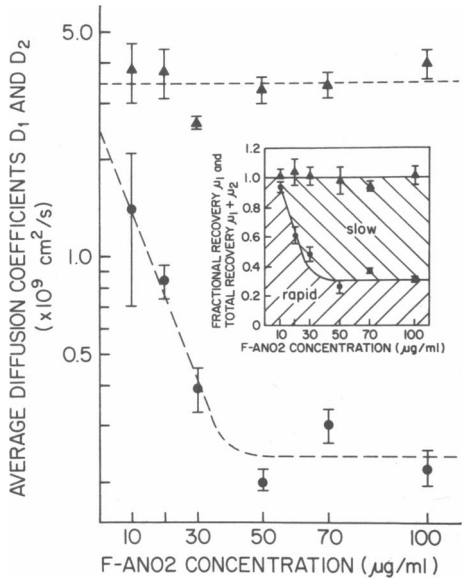

FIGURE 5 Average translational diffusion coefficients of F-AN02 on supported phospholipid bilayers. Shown are the values of the average diffusion coefficients D_1 (▲) and D_2 (●) and the average fractional recovery μ_1 (●) and the average total recovery $\mu_1 + \mu_2$ (▲) obtained from the best fits of the fluorescence recovery curves to the functional form expected for two mobile species (Eq. 2, $n = 2$). The average fractional recovery μ_2 equals $\mu_1 + \mu_2$ (▲) $- \mu_1$ (●) for each sample. The mean values and standard errors of the mean for 11–13 measurements per antibody concentration are shown.

due to the rapidly mobile component for 10-μg/ml samples is somewhat lower than for the higher concentrations. This intensity is constant within experimental uncertainty for solution concentrations of F-AN02 ≥ 20 μg/ml. Additional intensity ($\mu_2 F[t < 0]$) at the higher concentrations is associated with the slower population.

The average diffusion coefficient of NBD-PE in DMPC:NBD-PE bilayers was measured to be 2.6 ± 0.2 × 10^{-8} cm^2/s, which agrees with published results (Tamm and McConnell, 1985). Addition of 32 mol% DNP-TEMPO-DMPE to bilayers reduced the lipid diffusion coefficient by a factor of 6–7, to (3.7 ± 0.5) × 10^{-9} cm^2/s. The diffusion coefficient of NBD-PE in 32 mol% DNP-TEMPO-DMPE bilayers treated with 100 μg/ml unlabeled AN02 was measured to be (4.8 ± 0.4) × 10^{-9} cm^2/s, suggesting that the phospholipid bilayer structure was not significantly altered by bound antibodies. All fluorescence recovery curves for NBD-PE were fit well by the single-component model function given by Eq. 1.

The possibility that the nonmonophasic fluorescence recovery curves arose from an artifact of the experimental apparatus (e.g., nonuniformities of the Ronchi ruling) has been eliminated because data obtained for 10-μg/ml F-AN02 samples and for the fluorescent lipid probe NBD-PE in the supported bilayers were well fit by single-component functions. The possibility that the nonmonophasic behavior arose from a process other than translational diffusion (e.g., rotational diffusion) has been eliminated because significant recovery was not observed in the absence of the Ronchi ruling.

The measured values of D_1, D_2, μ_1, and μ_2 did not change within experimental uncertainty when the bleach pulse duration was doubled. However, this does not entirely eliminate the possibility that the photobleaching process induced covalent bonds between antibodies, forming aggregates and accounting for some or all of the slower diffusive population. Thus, experiments in which bound F-AN02 were bleached, recovered, and analyzed for covalent aggregates were performed (see Materials and Methods).[2] The results of the quantitative analysis of photobleached F-AN02 by SDS-PAGE indicated that 92 ± 12% of the surface-bound F-AN02 was recovered in monomeric form after photobleaching, which eliminated light-induced formation of antibody aggregates as a possible artifact. This result is consistent with a previous study (Sheetz and Koppel, 1979) in which substantial photoinduced cross-linking of fluorescein-labeled cell membrane proteins occurred only for exposures more than two orders of magnitude longer than those used in this work, at comparable intensities. The possibility that large noncovalent aggregates of F-AN02 form at the bilayer surface but are bound to the bilayer by only a small number of specific interactions is thought unlikely, but cannot be definitely eliminated.

The diffusion of AN02 antibodies on supported DMPC monolayers containing a haptenated lipid similar to the one shown in Fig. 1 has previously been investigated (Subramaniam et al., 1986). The films differed from those studied here in several ways. They were monolayers constructed on alkylated silicon/silicon dioxide substrates rather than bilayers on fused silica substrates; the DMPC films contained 5 mol% of a haptenated lipid with a 16-carbon chain length rather than 32 mol% of a haptenated lipid with a 14-carbon chain length; and the antibody incubation time was five rather than 20 min. Subramaniam and co-workers found that the diffusive behavior of antibodies bound to DMPC monolayers depended on the temperature, the chain length of the alkylating reagent, and the concentration of antibody with which monolayers were incubated. Although differences in the magnitudes of the antibody diffusion coefficients occurred, biphasic behavior was not observed. In addition, fractional mobilities as low as 0.1 were reported.

The diffusive behavior of a mouse antitrinitrophenyl IgG2b monoclonal antibody on DMPC plus trinitropheny-

[2]In these experiments, unlabeled sheep IgG was not added to the F-AN02 solutions, which would have made quantitative analysis of recovered antibodies difficult. The average background-corrected fluorescence intensities measured for haptenated bilayers treated with 100 μg/ml F-AN02 was, on the average, independent of the absence or presence of unlabeled sheep IgG. The ratios of measured intensities for bilayers containing haptenated lipids to those not containing haptenated lipids, and treated with 100 μg/ml F-AN02 with and without unlabeled sheep IgG, were not significantly different.

lated egg phosphatidylethanolamine bilayers supported on silicon/silicon dioxide substrates has very recently been described (Tamm, 1988). The samples differed from those of the present work not only chemically and physically but also kinetically in that bilayers were incubated with antibody solutions for five and not 20 min. Above the DMPC phase transition temperature, bound antibodies diffused rapidly and the fractional mobility ranged from 80 to 64% with increasing antibody surface concentration. Diffusive behavior for high molar fractions of haptenated lipids with high antibody surface concentrations were not reported. Below the DMPC phase transition temperature, antibody clusters larger than optical resolution were visually apparent as viewed by fluorescence microscopy.

The motions, interactions, and organizations of molecules in or on phospholipid Langmuir-Blodgett films are sensitive functions of the chemical, physical, and kinetic states of the films (Thompson and Palmer, 1988; McConnell et al., 1986). Thus, direct comparison of our results with those of Subramaniam et al. (1986) and Tamm (1988) is necessarily speculative. The most striking difference in observed behavior is that, in the present work, (*a*) mobility was distinctly biphasic, and (*b*) all of the bound antibodies were laterally mobile. The differences cannot be attributed solely to the particular antibody or haptenic head group, which is in common with Subramaniam et al. (1986), or solely to the use of bilayers rather than monolayers, which is in common with Tamm (1988). One common difference is the time with which bilayers were allowed to interact with antibodies. Because typical incubation times employed for antibody crystallization in Langmuir-Blodgett films are several hours (Uzgiris and Kornberg, 1983; Uzgiris, 1986), it is possible that the samples described in the present work are in a different kinetic state than those described by Subramaniam et al. (1986) and Tamm (1988). The films are not necessarily in an equilibrium state in any of the systems. Another common difference is that the films described in the present work contained a high molar fraction of a haptenated lipid with saturated acyl chains. Both of these factors might promote domain or cluster formation.

Theories that describe the concentration dependence of macromolecular diffusion in a homogenous medium, due to free volume accessibility (O'Leary, 1987), diffusion on a lattice (Kehr et al., 1981) or empirical considerations (Phillies, 1986) predict a smooth decrease in the diffusion coefficient with increasing concentration, not the appearance of a second diffusing population, and do not explain the reported observations. Thus, the possible coexistence of different regions in the bilayers must be considered, even though both DMPC and DNP-TEMPO-DMPE have 14-carbon acyl chains, the reported observations were above the chain melting temperature of DMPC and the bilayers appeared uniformly fluorescent under epi-illumination.

One possible explanation for the slower component would be coexistent domains of different local viscosities in which antibodies are independently mobile with different diffusion coefficients. If antibodies preferentially associate with DNP-TEMPO-DMPE in the less viscous domains, then at saturation a larger fraction of antibodies would be bound to DNP-TEMPO-DMPE in the more viscous domains and a larger fractional fluorescence recovery for the slower component would be observed. Immiscible domains in fluid vesicular bilayers of binary phospholipid composition (Melchior, 1986) and in cholesterol-containing DMPC monolayers at the air/water interface (Subramaniam and McConnell, 1987) have recently been reported. A second possibility is that bound antibodies induce the formation of domains of high local viscosity, in which the diffusion of antibody-lipid complexes is substantially restricted. Decreased membrane fluidity as measured by pyrene excimer fluorescence has been correlated with antibody binding to hapten-conjugated phospholipids in vesicles (Hart et al., 1985). However, fluorescence recovery curves of NBD-PE in DMPC: DNP-TEMPO-DMPE bilayers were well fit by the single-component function both in the absence and presence of bound unlabeled AN02. Thus the putative, more viscous lipid domains would have to exclude NBD-PE but not complexes of DNP-TEMPO-DMPE and F-AN02. In addition, because fluorescence pattern photobleaching recovery measures long-range diffusion and because two diffusive antibody components were reproducibly observed, the domains would have to be larger than the ruling periodicity in the sample plane (16 μm) but much smaller than the illuminated region (160 μm), and should have been microscopically visible due to the exclusion of NBD-PE (McConnell et al., 1986).

A third possible explanation is that the association of antibodies with DNP-TEMPO-DMPE induces the formation of submicroscopic antibody-containing domains (clusters). Such clusters might arise from direct antibody–antibody interactions, from antibody-induced lipid–lipid interactions, or from both. If the putative clusters had an appreciable diffusion rate within the remaining DMPC:DNP-TEMPO-DMPE matrix, they could directly account for the observed slower component. A final possibility is that antibodies bound to bilayers may associate into immobile submicroscopic clusters. If bound, clustered antibodies were in dynamic equilibrium with bound, monomeric antibodies, the second component could reflect the residency time of antibodies in immobile clusters (Koppel, 1981; Elson, 1985). The putative antibody clusters (mobile or immobile) might act as nucleation sites and precede crystallization of antibodies on phospholipid Langmuir-Blodgett films (Uzgiris and Kornberg, 1983; Uzgiris, 1986).

Clustering of ligand-receptor complexes on cell surfaces is an initial molecular event in cellular response to hormones (Cuatrecasas, 1983), growth factors (Yarden and Schlessinger, 1987) and immunochemicals (Davies and Metzger, 1983). In particular, clustering of antigen-

antibody-antibody receptor complexes (Metzger, 1983; Davies and Metzger, 1983; McCloskey and Poo, 1984), or, at the minimum, cross-linking of the receptors (Nitta et al., 1984; Young et al., 1983*a* and *b*) in the region of contact between immune effector cells such as macrophages or mast cells and their targets may be an important initial event in cellular activation. Cluster formation could occur as a result of the diffusive accumulation, and subsequent increase in concentration, of complexes of antigens, antibodies, and antibody receptors in the region of membrane–membrane contact (Balakrishnan et al., 1982*b*; McCloskey and Poo, 1984). If the observations reported in this work indeed reflect spontaneous clustering of antibodies on model membranes, then the possibility arises that clustered antibodies might form on intact cell surfaces and be related to subsequent effector cell activation.

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REFERENCES

Ameloot, M., and H. Hendrickx. 1982. Criteria for model evaluation in the case of deconvolution calculations. *J Chem. Phys.* 76:4419–4432.

Andries, C., W. Guedens, J. Clauwaert, and H. Geerts. 1983. Photon and fluorescence correlation spectroscopy and light scattering of eye-lens proteins for moderate concentrations. *Biophys. J.* 43:345–354.

Anglister J., T. Frey, and H. M. McConnell. 1984. Magnetic resonance of a monoclonal anti-spin label antibody. *Biochemistry.* 23:1138–1142.

Balakrishnan, K., F. J. Hsu, D. G. Hafeman, and H. M. McConnell. 1982*a*. Monoclonal antibodies to a nitroxide lipid hapten. *Biochim. Biophys. Acta.* 721:30–38.

Balakrishnan, K., F. J. Hsu, A. D. Cooper, and H. M. McConnell. 1982*b*. Lipid hapten containing membrane targets can trigger specific immunoglobulin E dependent degranulation of rat basophil leukemia cells. *J. Biol. Chem.* 257:6427–6433.

Beck, K. 1987. Mechanical concepts of membrane dynamics: diffusion and phase separation in two dimensions. *In* Cytomechanics. J. Bereiter-Hahn, O. R. Anderson, and W.-E. Reif. editors. Springer-Verlag, Berlin. 79–99.

Cuatrecasas, P. 1983. Developing concepts in receptor research. *Drug Intell. Clin. Pharm.* 17:357–366.

Davies, D. R., and H. Metzger. 1983. Structural basis of antibody function. *Annu. Rev. Immunol.* 1:87–117.

Devore, J. L. 1982. Probability and Statistics for Engineering and the Sciences. Brooks/Cole Publishing Co., Monterey, CA. 624.

Elson, E. L. 1985. Fluorescence correlation spectroscopy and photobleaching recovery. *Ann. Rev. Phys. Chem.* 36:379–406.

Hackenbrock, C. R., B. Chazotte, and S. S. Gupte. 1986. The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport. *J. Bioenerg. Biomembr.* 18:331–368.

Hart, M. J., K. Kimura, and M. Nakanishi. 1985. Selected positions of acyl chains are affected differently by antibody binding which results in decreased membrane fluidity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 190:249–252.

Kehr, K. W., R. Kutner, and K. Binder. 1981. Diffusion in concentrated lattice gases. Self-diffusion of noninteracting particles in three-dimensional lattices. *Physical Rev. B.* 22:4931–4945.

Koppel, D. E. 1981. Association dynamics and lateral transport in biological membranes. *J. Supramol. Struct. Cell. Biochem.* 17:61–67.

McCloskey, M., and M.-M. Poo. 1984. Protein diffusion in cell membranes: some biological implications. *Int. Rev. Cytol.* 87:19–81.

McConnell, H. M., T. H. Watts, R. M. Weis, and A. A. Brian. 1986. Supported planar membranes in studies of cell-cell recognition in the immune system. *Biochim. Biophys. Acta.* 864:95–106.

Melchior, D. L. 1986. Lipid domains in fluid membranes: a quick-freeze differential scanning calorimetry study. *Science (Wash. DC).* 234:1577–1580. Erratum, 238:550.

Metzger, H. 1983. The receptor on mast cells and related cells with high affinity for IgE. *Contempor. Top. Mol. Immunol.* 9:145–155.

Mishell, B. B., and S. M. Shiigi. 1980. *Selected Methods in Cellular Immunology.* W. H. Freeman and Co., San Francisco. 292–297.

Nitta, T., T. Saito-Taki, and T. Suzuki. 1984. Phospholipase A_2 activity of Fcγ2b receptors of thioglycollate-elicited murine peritoneal macrophages. *J. Leukocyte Biol.* 36:493–504.

O'Leary, T. J. 1987. Concentration dependence of protein diffusion. *Biophys. J.* 52:137–139.

Palmer A. G., and N. L. Thompson. 1987. Molecular aggregation characterized by high order autocorrelation in fluorescence correlation spectroscopy. *Biophys. J.* 52:257–270.

Phillies, G. D. J. 1986. Universal scaling equation for self-diffusion by macromolecules in solution. *Macromolecules.* 19:2367–2376.

Place, J. F., R. M. Sutherland, and C. Dahne. 1985. Opto-electronic immunosensors: a review of optical immunoassay at continuous surfaces. *Biosensors.* 1:321–353.

Ratkowsky, D. 1983. Nonlinear Regression Modeling. Marcell Dekker Inc., New York. 143–151.

Sheetz, M. P., and D. E. Koppel. 1979. Membrane damage caused by irradiation of fluorescent concanavalin A. *Proc. Natl. Acad. Sci. USA* 76:3314–3317.

Smith, B. A., and H. M. McConnell. 1978. Determination of molecular motion in membranes using periodic pattern photobleaching. *Proc. Natl. Acad. Sci. USA.* 75:2759–2763.

Subramaniam, S., and H. M. McConnell. 1987. Critical mixing in monolayer mixtures of phospholipid and cholesterol. *J. Phys. Chem.* 91:1715–1718.

Subramaniam, S., L. K. Tamm, N. L. Thompson, and H. M. McConnell. 1985. Fluorescence microscope studies of antibodies bound specifically to planar supported bilayers containing lipid haptens. *Biophys. J.* 47:367*a* (Abstr.).

Subramaniam, S., M. Seul, and H. M. McConnell. 1986. Lateral diffusion of specific antibodies bound to lipid monolayers on alkylated substrates. *Proc. Natl. Acad. Sci. USA* 83:1169–1173.

Tamm, L. K. 1988. Lateral diffusion and fluorescence microscope studies on a monoclonal antibody specifically bound to supported phospholipid bilayers. *Biochemistry.* 27:1450–1457.

Tamm, L. K., and H. M. McConnell. 1985. Supported phospholipid bilayers. *Biophys. J.* 47:105–113.

Thompson, N. L., and A. G. Palmer. 1988. Model membranes on planar substrates. *Comments Mol. Cell. Biophys.* 5:39–56.

Thompson, N. L., A. A. Brian, and H. M. McConnell. 1984. Covalent linkage of a synthetic peptide to a fluorescent phospholipid and its incorporation into supported phospholipid monolayers. *Biochim. Biophys. Acta.* 722:10–19.

Ullmann, K., G. S. Ullmann, and G. D. J. Phillies. 1985. Optical probe study of nonentangling macromolecule solution—bovine serum albumin: water. *J. Colloid Interface Sci.* 105:315–324.

Uzgiris, E. E. 1986. Supported phospholipid bilayers for two-dimensional

protein crystallization. *Biochem. Biophys. Res. Commun.* 134:819–826.

Uzgiris, E. E., and R. D. Kornberg. 1983. Two-dimensional crystallization technique for imaging macromolecules, with application to antigen-antibody-complement complexes. *Nature (Lond.)* 301:125–129.

Yarden, Y., and J. Schlessinger. 1987. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry.* 26:1434–1451.

Young, J. D., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983*a*. Mouse macrophage Fc receptor for IgGγ2b/γ1 in artificial and plasma membrane vesicles functions as a ligand dependent ionophore. *Proc. Natl. Acad. Sci. USA.* 80:1636–1640.

Young, J. D., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983*b*. Macrophage membrane potential changes associated with γ2b/γ1 Fc receptor ligand binding. *Proc. Natl. Acad. Sci. USA.* 80:1357–1361.

Zemel, J. N., editor. 1983. *Thin Solid Films 99.* Proceedings of the First International Conference on Langmuir-Blodgett Films. 329 pp.